Formulating microbial biocontrol agents†

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Abstract: Micro-organisms can be used to provide a crop protection effect, but to exploit this it is necessary to deliver viable micro-organisms as a formulated product suitable for agronomic application, which presents a significant challenge to formulation technology.

Gram-negative bacterial cells can be formulated in a dry form which is shelf-stable at elevated temperatures for periods exceeding six months. This paper presents the key physical requirements of the dried formulation required to generate this stability, and a predictive mathematical model of the kinetics of microbial degradation as a function of key physical parameters, for example storage temperature and relative humidity.

Keywords: microbial; stabilisation; biocontrol agent; glass transition

1 INTRODUCTION

Microbial agents, for example bacteria, fungi, viruses, have been shown to have intrinsic potential as agents for crop protection. However, to be of practical use they must be formulated as products capable of storage, distribution and application into the agricultural marketplace, requiring different approaches from traditional agrochemical product design.

A particular challenge is to stabilise vegetative bacterial cells for extended shelf life without compromising application and activity in the field.

1.1 Stabilisation at reduced metabolic activity

Adequate shelf life for a product in this market requires stability over at least a one-year period. Vegetative bacteria require a steady supply of nutrient and removal of toxic metabolites from the stored product, and it is impractical to design a product which could supply nutrient for even low levels of metabolic activity over this time period. Therefore it is necessary to develop a formulation which reduces the metabolic activity to negligible levels without killing the cell.

The incidence of cell death can be minimised by growing the cells in complex media,[1] optionally under conditions of high ionic strength,[2] or in the presence of selected ions,[3] harvesting at early stationary phase of growth,[4] and drying them in the presence of suitable protective agents.[5]

1.2 Storage stability

A more complex problem is to maintain the stability during extended periods of storage over the range of temperature that a product is likely to experience in the distribution chain. Previous workers have shown that storage life is also sensitive to growth stage at harvesting,[6] ionic composition of the solution used to resuspend cells prior to drying[7] and choice of protective agent.[8]

However, the work reported below shows that the physical state of the matrix is also critical to the stabilisation of viable cells.

2 EXPERIMENTAL

Pseudomonas fluorescens (Trevisan) Migula, an antifungal strain of bacteria isolated from soil (isolate identity ZA54/96), were grown in double-strength nutrient broth in 200 ml shake-flask culture at 20°C, or in 10-litre fermenters, harvested by centrifugation at early stationary phase of growth (typically 24 h after inoculation), resuspended in a solution of rhamnose (a monosaccharide) and sodium ascorbate (to ratio of 10 or 100 mg saccharide: 10 mg sodium ascorbate: 1×10^{10} cells: 1 ml water). This slurry was poured into a tray to a depth of between 2 mm and 1 cm, and either vacuum dried at ambient temperature for 10–24 h, or freeze-dried with temperature cycle of primary drying at shelf temperature − 30°C for 48 h at mean pressure of 0.01 mbar, and

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secondary drying at 0°C for 24 h at mean pressure of 0.005 mbar.

Samples were transferred to vacuum-sealed foil bags and stored either in temperature-controlled ovens or in open bags at 20(±2)°C under a stream of controlled-humidity air. Glass transition temperatures (T_g) of dried materials were measured on a Perkin-Elmer differential scanning calorimeter at scanning rates of 5°C min^{-1} and T_g values taken from the mid-point of the second-order transition curve.

Cell viability was determined by resuspension in sterile water followed by serial dilution in sterile water: five replicate samples were plated on to King's B Agar and colonies were counted after incubation at 20°C for 48 h.

Survival figures quoted are averages of two rehydrated replicate samples, each dispersion being sampled for serial dilution once, and cell counts determined as an average of five replicate plate counts. Variation in the viable cell count was used to estimate typical uncertainty values in the first-order rate constant k (see below).

3 RESULTS AND DISCUSSION

3.1 Storage lifetime dependence on the glass transition of the matrix

The rate of loss of cell viability has been found to be approximately first-order with time, which has allowed the development of a simple phenomenological model to predict storage lifetime.

The dried formulation matrix, to some degree, acts as a diffusion barrier, reducing the diffusion (D) of reactive species which can give rise to cell death. In effect:

$$\text{rate of cell death} \propto k_1\,[\text{Live cell}]$$

where k_1 is effectively a first-order rate constant. Since the matrix is essentially amorphous, the diffusion can be treated, to a first approximation, as movement through a viscous liquid i.e.:

$$k \propto D,$$

where;

$$D = kT/6\pi a\eta$$

Where the viscosity, η, is given by the Williams Landel Ferry[9] equation:

$$\log\,(\eta/\eta_r) = -c_1(T_s - T_g)/[c_2 + (T_s - T_g)],$$

i.e.

$$\log\,(k_1/k_r) = c_1'(T_s - T_g)/[c_2' + (T_s - T_g)]$$

Therefore, one would expect the rate constant of cell degradation (k_1) to show a step change increase if the storage temperature (T_s) exceeds the glass transition temperature (T_g) of the formulation, as is seen in Fig 1.

Similarly, since the matrix is hydrophilic, the relative humidity of the air to which the sample is exposed during storage will affect the matrix composition and, by plasticising the matrix, reduce the glass transition temperature, increasing the rate of cell death, as seen in Fig 2.

3.2 Storage lifetime dependence on matrix physical form

The physical form of the formulated material is also important to good storage stability. The matrix can be made to undergo viscous flow (collapse in freeze-drying terminology), by which the material 'shrink-wraps' the cells. This reduces the effective surface area by which oxygen or water can diffuse into the matrix, and reduces the rate of cell death, as shown in Figs 1 and 2, where the higher saccharide concentration allows collapse to occur.

3.3 Matrix composition and the crop protection effect

The initial product using this technology is a fungicidal bacterium for soil use. A limitation of carbohydrate-based formulations is the potential to stimulate growth of the fungal soil pathogen. Having established the physical requirements of the matrix to deliver storage stabilisation and good resuspension properties using the rhamnose-based formulation, the system was then optimised using a non-nutrient hydrophilic polymer, polyvinylpyrrolidone containing urea and sodium ascorbate. The final composi-

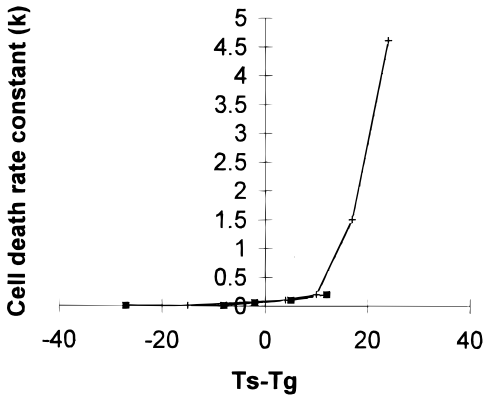

Figure 1. First-order rate constant (k) of *Pseudomonas* cell death during storage of freeze-dried formulations containing rhamnose and sodium ascorbate mixtures, where (■) the freeze-dried matrix has collapsed (100 mg rhamnose per 10^{10} cells) or (+) has not been allowed to collapse (10 mg rhamnose per 10^{10} cells). Samples were stored in sealed foil bags at fixed moisture content, at temperatures (T_s) between 5°C and 45°C. When the storage temperature exceeds the glass transition temperature (ie positive values of the x-axis), the rate of cell death (k) increases to a much greater extent than would be expected from simple Arrhenius kinetics. Typical uncertainty in k values ±0.05: typical uncertainty in $T_s - T_g$ ± 10°C.

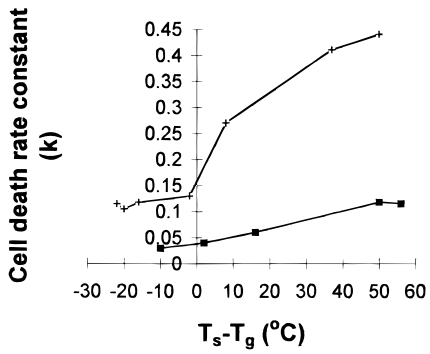

Figure 2. First-order rate constant (k) of *Pseudomonas* cell death during storage of freeze-dried formulations containing rhamnose and sodium ascorbate mixtures, where (■) the freeze-dried matrix has collapsed (100 mg rhamnose per 10^{10} cells) or (+) has not been allowed to collapse (10 mg rhamnose per 10^{10} cells). Samples were stored at 21°C (T_s), exposed to a flow of air of controlled relative humidity (between 2% and 55% RH). Moisture adsorbed from the air reduces the glass transition temperature (T_g) of the dried materials. When the glass transition of the material is below the storage temperature (ie $T_s - T_g$ is positive), the material is in a rubbery state, and the rate of cell death (k) is significantly greater than when the material is in a glassy state (ie when $T_s - T_g$ is negative). Collapsed material (■) is more stable than uncollapsed material (+) in both the rubbery and glassy states. Typical uncertainty in k values ±0.02: typical uncertainty in $T_s - T_g$ ±10°C.

tion could then be optimised to deliver excellent temperature stability and significant robustness to moisture content. In extended stability tests, vacuum-dried material with this composition stored in foil sachets, showed retention of >50% of viable cells after six months' storage at 40°C.

4 CONCLUSIONS

It is possible to stabilise vegetative bacterial cells in a dried form by vacuum drying or freeze-drying, without significant loss of viable cells either during preparation or during storage.

The kinetics of bacterial loss can be quantified as approximately first-order, and the rate constant of cell death exhibits a step change increase above the glass transition temperature of the matrix. This provides a predictive model of shelf life as function of storage temperature and moisture content.

The formulation design can be optimised to deliver extended shelf life by controlling the physical form and chemical composition of the matrix.

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